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Comparison of the Sensitivities of Noroviruses and Feline Calicivirus to Chemical Disinfection under Field-Like Conditions▽

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Noroviruses (NV), in the family *Caliciviridae*, are an important cause of gastroenteritis in humans worldwide. Measures for prevention and control of NV dissemination are therefore necessary to ensure public safety. The abilities of an organic acid (Venno Vet 1 Super), an aldehyde (Venno FF Super), a halogen compound (sodium hypochlorite solution), and a peroxide (Oxystrong FG) to inactivate feline calicivirus (FCV), a cultivable virus surrogate for NV, were studied. Molecular protocols were then used for the comparative evaluation of disinfectant efficacies against NV and FCV, which were tested by reproducing NV field conditions, using human fecal material as a protein load. Generally, disinfectant efficacy was strongly reduced by the organic impurities (feces) used during tests. All disinfectants, except the aldehyde, were effective on FCV, as measured by cell culture and reverse transcription-PCR (RT-PCR), with inactivation levels of ≥99.9%. The glutaraldehyde-based compound failed to adequately inactivate FCV according to RT-PCR results, although the infectivity in cell culture was completely abolished. Similar inactivation levels were achieved with NV, but generally NV appeared more resistant than FCV, and consequently, the suitability of FCV as a model for NV should be considered with caution. In conclusion, according to RT-PCR results, 5% Venno Vet 1 Super, 1% Oxystrong FG, and not less than 2% Venno FF Super, with a contact time of 1 h, and 1% sodium hypochlorite, with 6,000 ppm of free chlorine and a contact time of 15 min, are required for safe disinfection when a calicivirus-related outbreak is suspected.

Members of the family *Caliciviridae* are nonenveloped small viruses with single-stranded positive-sense RNA genomes. The family is currently divided into the following four genera: *Norovirus*, *Sapovirus*, *Vesivirus*, and *Lagovirus* (9, 12).

Among these viruses, noroviruses (NV) have emerged as one of the most common causes of acute gastroenteritis in humans, with sporadic cases as well as large outbreaks occurring in various community settings (11, 25). During surveillance of viral gastroenteritis outbreaks in Europe, around 90% of nonbacterial outbreaks could be attributed to NV (25, 42), while NV was responsible for 96% of nonbacterial outbreaks in the United States (10) and a similar high prevalence was reported in studies from Japan and Australia (11).

Although the course of disease is usually mild and self-limiting, the combination of high viral loads in vomit and feces together with a low minimal infective dose and high environmental stability of the viral particles represents a public health risk (17, 19). In addition, severe disease with dehydration, hospitalization, and risk of fatal outcome can also develop, particularly in elderly and immunocompromised or weakened patients (11). NV infection can be spread by person-to-person contact, contaminated food or water, aerosol, and environmental surfaces, such as toilets (21, 26). Previous studies have revealed the potential role of environmental surfaces in the transmission of caliciviruses (4, 13), and residual virus after disinfection can result in prolonged or recurring outbreaks with considerable economic losses (23). Therefore, measures for prevention and control of calicivirus spread are necessary to ensure public safety.

Since NV are very difficult to grow in cell culture and a cell culture system for the propagation of NV has only recently been described (8, 39), a closely related virus, the feline calicivirus (FCV) of the genus *Vesivirus*, has been suggested to be an adequate model for NV inactivation studies because it has a similar genome organization, capsid architecture, and biochemical properties (4, 6, 13, 31, 36, 40). FCV, one of the most common agents of conjunctivitis and upper respiratory tract disease in cats, can be cultured and plaque assayed (6, 30, 31, 36, 38, 40).

According to the guidelines of the German Veterinary Association (1), disinfection tests were carried out with four commercially available disinfectants (an organic acid [Venno Vet 1 Super], an aldehyde [Venno FF Super], a halogen compound [sodium hypochlorite solution {bleach}], and a peroxide [Oxystrong FG]) in order to determine their potential to accomplish the claims which are made regarding their efficacies under realistic test conditions. Since human fecal material is the realistic organic load in whose presence disinfectants should be effective against NV, disinfection tests were carried out on FCV by using a human stool sample as a suspending medium in order to reproduce NV environmental conditions. In this context, we evaluated the suitability of FCV as a model for NV, using comparable test conditions and molecular methods (reverse transcription-PCR [RT-PCR] assays) to test both cultivable and noncultivable viruses. Two different RT-PCR proto-

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TABLE 1. Disinfectants used during disinfection tests on FCV and NV

Disinfectant class	Product	Composition	Manufacturer
Organic acid	Venno Vet 1 Super	55 to 60% formic acid, 7% glyoxylic acid	Menno Chemie Vertrieb, Norderstedt, Germany
Aldehyde	Venno FF Super	20 to 25% glutaraldehyde, 12% oligomers	Menno Chemie Vertrieb, Norderstedt, Germany
Halogen compound	Sodium hypochlorite solution	12% sodium hypochlorite	Roth, Karlsruhe, Germany
Peroxide	Oxystrong FG	14 to 16% peracetic acid, 22 to 24% hydrogen peroxide, <15% acetic acid	Ausimont, Bitterfeld, Germany

cols were used for NV and FCV, and their sensitivities were analyzed and compared and their discrepancies, based upon different target sequences, evaluated. This was also done using a quantitative real-time PCR performed using a LightCycler technique with corresponding software.

MATERIALS AND METHODS

Viruses and cells. FCV strain F9 (Virbagen-felis RC; Virbac SA, Carros, France) was propagated in Crandell Reese feline kidney cells (CCL-94; American Type Culture Collection, Wesel, Germany) cultured in Dulbecco's modified Eagle's medium (DMEM) (all reagents for cell culture were provided by Biochrom AG, Berlin, Germany) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS). Virus was added to cell monolayers in 175-cm^2 disposable tissue culture flasks (Nunc, Roskilde, Denmark) and allowed to adsorb for 1 h at 37°C in an atmosphere of 5% CO_2 and >90% relative humidity, and then medium was added and the flasks again incubated as before. The maintenance medium for virus propagation was DMEM with 2% (vol/vol) FBS, penicillin G (200 U/ml), streptomycin sulfate (0.5 mg/ml), and amphotericin B (1 μg/ml). The cells were monitored for cytopathic effect, and the stock virus was harvested after one freeze-thaw cycle (at −80°C) by transferring the medium with the cells into 50-ml plastic centrifuge tubes and centrifuging them at about 1,600 × *g* for 15 min at 4°C. The supernatant was decanted and stored at −80°C in 1-ml aliquots. The virus pool supernatant from infected monolayers was titrated by end-point titration in cell culture and by RT-PCR to estimate the end point of serial 10-fold dilutions resulting in positive amplification products.

A stool specimen positive for NV (genogroup II) (18), provided by the Landesgesundheitsamt Baden-Württemberg (Stuttgart, Germany), was prepared as a 50% (wt/vol) suspension in water of standardized hardness (pH 7.2) and titrated by conventional nested RT-PCR and real-time PCR. The virus was stored in 1-ml aliquots at −80°C.

Stool specimens were collected from persons without reported gastrointestinal illness and tested by nested RT-PCR for NV infection and by RT-PCR for FCV contamination. Negative samples were stored at −80°C in 1-g aliquots and used as the organic load for FCV disinfection tests. FCV stock aliquots were mixed 1:1 with fecal aliquots in a 50% (wt/vol) suspension, diluted 10-fold in DMEM, and titrated by cell culture, conventional RT-PCR, and real-time PCR.

Virus quantitation by cell culture. The infectivity titers of FCV from stock aliquots and inactivated mixtures were determined as the 50% tissue culture infective doses ($TCID_{50}$) per milliliter according to the method of Spearman and Kärber (15). Crandell Reese feline kidney cell suspensions in DMEM with 10% FBS, seeded in 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 3×10^4 cells per well, were infected by the addition of 100 μl of serial 10-fold dilutions of virus (in maintenance medium) per well, with four replicates per dilution. Plates were incubated for 10 days at 37°C in an atmosphere of 5% CO_2 and >90% relative humidity.

Disinfection tests. The disinfectants tested are listed in Table 1 and include an organic acid, an aldehyde, a halogen compound, and a peroxide. Suspension tests were carried out on FCV, with and without protein load (40% FBS) and in a 25% fecal suspension (wt/vol), according to the guidelines of the German Veterinary Association (1, 2). Known quantities of virus suspensions were mixed and incubated with the disinfectants (diluted in water of standardized hardness) or with DMEM as a test control at 20°C for defined exposure times (15, 30, 60, and 120 min). The virus-disinfectant mixtures (and the virus-control mixtures) were then detoxified by dilution in phosphate-buffered saline (pH 7.5) before cell culture titration. The concentrations that were found to be effective or highly effective were chosen for suspension tests on FCV again and for molecular titration on NV. The four highest 10-fold dilutions of the untreated virus (FCV or NV) that were positive in the nested RT-PCR were examined after disinfection tests. Disinfection tests were carried out in duplicate to confirm disinfectant efficacy, and results were subjected to statistical analysis through one-way analysis of variance procedures and analyses of the least significant difference according to the method of Montgomery (28).

During disinfection tests with sodium hypochlorite solution, the amount of free chlorine was calculated approximately through iodometric titration for each concentration used.

"Pretreatment" with PK and RNase. The FCV RT-PCR commonly yielded positive results with samples that failed to yield virus in culture and therefore contained only inactivated virus. Pretreatment with proteinase K (PK) and RNase, developed by Nuanualsuwan and Cliver (29) in order to eliminate the RNAs of inactivated viruses (such as inactivated FCV), was attempted after some disinfection tests with the organic acid and the aldehyde.

Virus quantitation by molecular methods. Viral RNAs were extracted from serial 10-fold dilutions of virus suspensions (FCV and NV) before and after disinfection, using the guanidinium isothiocyanate method described by Boom et al. (5). Primers employed by Scansen et al. (33) were selected for FCV PCR, which was performed in a single round. For NV, primers designed by Schreier et al. (34) were chosen, and the published nested RT-PCR protocol was followed. Both primer sets (MWG-Biotech AG, Ebersberg, Germany) target conserved portions in ORF1 (the FCV primers target the p30 gene, and the NV primers target the RNA-dependent RNA polymerase gene) (Table 2).

(i) RT. For both caliciviruses, cDNA was generated from 5 μl of viral RNA by using 30 pmol reverse primer (FCVp30 rev or NV7) in a 20-μl mixture containing 1 mM of each deoxynucleoside triphosphate (dNTP) (peqGold dNTP set; peqLab Biotecnologie, Erlangen, Germany), 40 U RNase inhibitor (MBI Fermentas, Germany), 160 U reverse transcriptase, and 4 μl 5× associated buffer

TABLE 2. FCV and NV primers and amplicons

Virus	PCR method	Primer direction	Primer name, sequence (5′–3′)	ORF1 nt[a]	Amplicon size (bp)
FCV	Real-time RT-PCR	Forward	FCVp30, TGG ATG AAC TAC CCG CCA	2415–2540	126
	Real-time RT-PCR	Reverse	FCVp30, GCA CAT CAT ATG CGG CTC		
NV	RT-PCR	Forward	NV1a, ATG AAT ATG AAT GAA GAT GG	4226–4707	482
	RT-PCR	Reverse	NV7, ATT GGT CCT TCT GTT TTG TC		
	Nested real-time PCR	Forward	NV4, TAC CAC TAT GAT GCA GAT TA	4280–4617	338
	Nested real-time PCR	Reverse	NV6, GTT GAC ACA ATC TCA TCA TC		

[a] GenBank accession no. M86379 (for FCV) and X86557 (for NV).

TABLE 3. Effective disinfectant concentrations (and respective contact times), by cell culture and RT-PCR, against FCV in suspension tests under different test conditions

Disinfectant type	Effective conditions in suspension test[a]					
	Without protein		With 40% FBS		With 25% feces	
	Cell culture	RT-PCR	Cell culture	RT-PCR	Cell culture	RT-PCR
Organic acid	0.5%, 15 min	0.5%, 60 min	4%, 15 min	4%, 60 min	4%, 15 min	4%, 30 min
Aldehyde	0.1%, 15 min	—	0.1%, 15 min	—	0.1%, 15 min	—
Halogen compound	0.5% (4,500 ppm), 15 min	0.5% (4,000 ppm), 15 min	0.5% (4,500 ppm), 15 min	—	0.75% (5,500 ppm), 15 min	—
Peroxide	0.1%, 15 min	—	0.1%, 15 min	—	1%, 60 min	1%, 60 min

[a] Effective conditions led to inactivation levels of ≥99.9%, corresponding to ≥3-$\log_{10}$ titer reductions. —, no effect.

(RevertAid H^- Moloney murine leukemia virus reverse transcriptase; MBI Fermentas, Germany). RT was performed for 10 min at 25°C and 1 h at 42°C, and the reverse transcriptase was inactivated at 98°C for 5 min. The resulting cDNA was then amplified by PCR.

(ii) Conventional PCR. For each virus, the PCR assay was done using 2 μl from the RT reaction mix and 30 pmol of each sense and antisense primer (FCVp30 rev and forw or NV7 and NV1a) in a 50-μl (final volume) mixture containing dNTPs at a concentration of 0.125 mM, 2.5 U *Taq* DNA polymerase, 5 μl 10× buffer (MBI Fermentas, Germany), and 2 mM $MgCl_2$ (for FCV) or 1.25 mM $MgCl_2$ (for NV). The second round of NV PCR was performed like the first, using NV4 and NV6 as sense and antisense primers and mixing 1 μl of the first-round product with 49 μl of the PCR mixture, with the same concentrations of reagents. Amplification was carried out in an Omnigene Hybaid thermocycler (MWG-Biotech AG, Ebersberg, Germany), and the cycling conditions were as follows: initial heat denaturation at 94°C for 1 min; 35 cycles at 94°C for 30 s, 52°C (for FCV) or 42°C (for NV [first and second rounds]) for 30 s, and 72°C for 45 s; and a final extension at 72°C for 3 min.

Products (10 μl) from each RT-PCR were analyzed by gel electrophoresis on 1.5 to 2% agarose, stained with ethidium bromide, and visualized by UV illumination. The end point of detection for RT-PCR assays represents 1 RT-PCR detection unit (RTPCRU), and the total number of RTPCRU was calculated as the $\log_{10}$ RTPCRU per milliliter (7, 14, 16, 35).

(iii) Real-time PCR. Tenfold dilutions of virus stock aliquots (NV and FCV mixed 1:1 with feces) were subjected to real-time PCR. The FCV cDNA and the NV first-round PCR product, obtained as described above, were used as templates. Real-time PCR was carried out using a LightCycler DNA master SYBR green I kit and a LightCycler instrument with software (versions 5.32 and 3.5; Roche, Mannheim, Germany). Twenty-microliter reaction mixtures, assembled in glass capillaries, were made by combining 10 pmol of each primer (FCVp30 forw and rev or NV4 and NV6) with 2 μl of 10× SYBR green I master mix (containing *Taq* DNA polymerase, reaction buffer, dNTP mix, SYBR green I dye, and 10 mM $MgCl_2$), $MgCl_2$ at a final concentration of 3 mM, and 10.4 μl Millipore water and 2 μl cDNA (for FCV) or 11.4 μl Millipore water and 1 μl first-round PCR product (for NV). The thermal profile for the reactions consisted of a step of DNA template denaturation and simultaneous DNA polymerase activation at 95°C for 10 min followed by 35 cycles of 15 s at 95°C, 20 s at 52°C, and 10 s at 72°C for FCV and by 35 cycles of 15 s at 95°C, 20 s at 42°C, and 10 s at 72°C for NV. After amplification, for one cycle of melting curve analysis, the temperature was increased to 95°C, cooled and kept for 15 s at 65°C (NV) or 75°C (FCV), and again increased to 95°C. Finally, amplicons were subjected to a cooling cycle at 40°C for 30 s. The last dilution that produced a positive cycle threshold (C_T) value was designated 1 RTPCRU, and stock titers were expressed as the $\log_{10}$ RTPCRU per milliliter (14, 16). The specificity of the designed primers was tested because the amplicons were identified by melting curve analysis and confirmed by gel electrophoresis. Using external standard curves, NV and FCV template amplifications were compared by their respective C_T values, and differences between NV and FCV C_T values (ΔC_T) were considered (22, 32).

RESULTS

FCV quantitation. The standard microtitration assay yielded virus titers ranging from 8.5 to 9.35 $\log_{10}$ $TCID_{50}$/ml for FCV stock solutions, which corresponded to titers of 4 to 5 $\log_{10}$ RTPCRU/ml with conventional RT-PCR. FCV aliquots of 9.35 $\log_{10}$ $TCID_{50}$/ml mixed 1:1 with aliquots of a negative fecal sample in a 50% (wt/vol) suspension still demonstrated a titer of 5 $\log_{10}$ RTPCRU/ml by conventional RT-PCR and real-time PCR. The identification of a melting temperature of between 86 and 87°C by melting curve analysis and of bands of 126 bp by gel electrophoresis indicated specific products.

NV quantitation. The titer of NV in a 50% (wt/vol) fecal suspension in water of standardized hardness was 5 $\log_{10}$ RTPCRU/ml by conventional and real-time PCR. The specific PCR product of 338 bp, identified by gel electrophoresis, melted at a temperature of around 87°C. Considering the FCV and NV linear regression analyses where C_T values were plotted against the dilution factors, the average ΔC_T per 10-fold dilution corresponded to 3.4 cycles for FCV and 2.9 cycles for NV, and differences between the NV and FCV C_T values (ΔC_T), calculated for each positive 10-fold dilution (from 10^{-1} to 10^{-5}), were 1.032, 1.524, 2.016, 2.508, and 3, respectively. Therefore, the higher the dilution, the greater was the difference between the cycle numbers at which the fluorescence generated during FCV and NV amplification reached a specific threshold detection level, with lower values for NV. However, high FCV and NV concentrations showed very low ΔC_T values (~1).

Disinfection test results. The results of the disinfection tests are shown in Tables 3 to 5. Using the virus control as a reference, the level of inactivation was calculated as the $\log_{10}$ reduction of the titer, in $TCID_{50}$/ml or RTPCRU/ml. The criterion normally set for virucidal efficacy is a 99.9% (3 $\log_{10}$), 99.99% (4 $\log_{10}$), or 99.999% (5 $\log_{10}$) reduction in virus titer. A standard decrease of 3 $\log_{10}$ was considered adequate (13, 24, 37).

(i) Venno Vet 1 Super. In the absence of organic matter, a concentration of 0.5% Venno Vet 1 Super inactivated FCV by ≥5 $\log_{10}$ steps after only 15 min of contact (cell culture) or by 2 or 3 $\log_{10}$ steps after 15 min or 60 min (RT-PCR) (Table 3). In the presence of FBS or feces, a 0.5% suspension led, by cell culture, to a reduction of <1 $\log_{10}$ of the FCV titer, even after 120 min of contact, and a reduction of ≥4 dilution steps was achieved only with a 4% concentration, after 15 min of contact, or a 3% concentration, after 120 min. However, a concentration of 3% was effective, with around 3-$\log_{10}$ reductions of the FCV titer after 60 min of contact. By nested RT-PCR, 3 $\log_{10}$ steps of reduction were measured for FCV after 30 to 60 min of contact with a 4% concentration and for NV after 60 min with a 5% concentration, but a 3% concentration was ineffective (Tables 4 and 5).

TABLE 4. Virucidal efficacies of different concentrations of disinfectants against FCV and NV in suspension tests with human fecal material

Disinfectant type	Concentration (%)	FCV titer (log_{10} RTPCRU/ml) after indicated time (min)[a]				NV titer (log_{10} RTPCRU/ml) after indicated time (min)[a]			
		15	30	60	120	15	30	60	120
Organic acid	3	5	5	5	5	5	5	5	5
	4	3	**2**	**2**	**2**	4	4	4	4
	5	—	—	—	—	4	3	**2**	**2**
Aldehyde	0.1	5	4	5	5	5	5	5	5
	0.5	4	4	3	3	5	5	5	5
	1	—	—	—	—	4	4	4	4
	2	—	—	—	—	4	4	3	3
Halogen compound	1	5	5	5	5	4	3	3	4
	6,000 ppm free chlorine	**2**[b]	**2**[b]	**2**[b]	**2**[b]	**≤1**[b]	**2**[b]	**≤1**[b]	**2**[b]
	1.2	5	4	5	5	4	4	4	4
	7,000 ppm free chlorine	**2**[b]	**2**[b]	**≤1**[b]	**2**[b]	**≤1**[b]	**≤1**[b]	**≤1**[b]	**≤1**[b]
Peroxide	1	**2**	3	**2**	**2**	3	3	**2**	**2**
	2	**2**	**2**	**2**	**2**	3	3	**2**	**2**

[a] Values in bold indicate titer reductions of ≥3 log_{10}. The control titer was 5 RTPCRU/ml in all cases. —, not tested.
[b] There was 10% fecal material in the virus-disinfectant mixture.

(ii) Venno FF Super. In the cell cultures, 0.1% Venno FF Super reduced FCV titers >4 log_{10} or ≥3 log_{10} steps after only 30 min of contact in tests without proteins or with FBS, respectively. When tests were carried out with a protein source (FBS or feces), a 0.5% concentration produced ≥4-log_{10} reductions after 15 min. In contrast, a maximum of a 2-log_{10} reduction was obtained by RT-PCR with a 0.5% concentration of disinfectant, after a reaction time of 1 or 2 h, during tests without organic load or with feces (Table 3). A concentration of 0.5% was completely ineffective against NV, and a reduction of 2 log_{10} steps was detected only twice, with 2% mixtures, once after 2 h and once after 1 h of contact (Tables 4 and 5).

(iii) Venno Vet 1 Super and Venno FF Super disinfection with "pretreatment." FCV dilutions for which the RT-PCR gave positive results, even though cell culture tests were negative, still yielded positive bands of intact nucleic acid after treatment with PK and RNase prior to RNA extraction. Even with enzymatic digestion, disinfectant concentrations capable of highly efficacious inactivation when tested by cell culture did not lead to >2 log_{10} steps of titer reduction by RT-PCR. Treatments with PK and RNase were not successful in reducing the RT-PCR titers measured, even when cell culture results indicated that the detected RNAs might stem from inactivated virus particles.

(iv) Sodium hypochlorite solution. During tests without feces, 0.5% sodium hypochlorite (4,500 to 5,000 ppm of free chlorine) produced, by cell culture, >5-log_{10} reductions of the FCV titer after 15 min of contact, while 0.1% sodium hypochlorite (800 to 900 ppm of free chlorine) was effective only after 2 h. During tests with feces, drops of 4 log_{10} steps were detected with 0.75% of hypochlorite (5,500 ppm of free chlorine). By RT-PCR, a 0.1% concentration seemed to have no effect on FCV. Nested RT-PCR was also unable to detect any effect of 0.75%, 1%, and the highest available concentration of 1.2% sodium hypochlorite (5,500, 6,000, and 7,000 ppm of free chlorine, respectively) on FCV and NV in suspension tests with FBS or feces. A 0.5% concentration yielded ≥4-log_{10} reductions of FCV only in tests without protein load (FBS or feces) (Table 3). Furthermore, after reducing the percentage of fecal material in the viral suspension (from 25% to 10%), the same high chlorine concentrations, of 6,000 and 7,000 ppm, appeared immediately effective by molecular evaluation, resulting in 3- and ≥4-log_{10} reductions, respectively, of the NV or FCV titer (the control titer was still 5 log_{10} RTPCRU/ml, while inactivated virus titers decreased to 2 or ≤1 log_{10} RTPCRU/ml) (Tables 4 and 5).

(v) Oxystrong FG. In suspension tests without feces, >4-log_{10} reductions of the FCV titer were detected, by cell culture, with 0.1% Oxystrong FG, while a 0.5% concentration, under the same conditions and after 15 min of contact, produced >6-log_{10} reductions. By RT-PCR, 0.1% and 0.5% concentrations produced an immediate and consistent 2-log_{10} reduction of the FCV titer (from 5 to 3 log_{10} RTPCRU/ml). A 0.1% concentration was insufficient to oxidize viral components in

TABLE 5. Disinfectant concentrations (and contact times) associated with the highest reductions of the FCV and NV titers registered by RT-PCR

Disinfectant type	Reduction factor (log_{10})	Conditions in suspension tests with feces	
		FCV	NV
Organic acid	3	4%, 30 min	5%, 60 min
Aldehyde	2	0.5% 60 min	2%, 60 to 120 min
Halogen compound	≥3[a]	1% (6,000 ppm free chlorine), 15 min	1% (6,000 ppm free chlorine), 15 min
Peroxide	3	1%, 60 min, or 2%, 15 min	1%, 60 min, or 2%, 60 min

[a] During suspension tests with 10% feces.

suspension with feces; 0.5% and 1% concentrations were effective by cell culture, with reductions of only 3 $\log_{10}$ dilutions; and a 2% concentration was necessary to quickly and effectively disinfect FCV (after 15 min, no virus growth was detected). Molecular results showed that 1% and 2% concentrations reduced the FCV titer by 3 $\log_{10}$ steps (in fecal suspension) after 1 h and 15 min, respectively, and the NV titer after 1 h (Tables 4 and 5).

Statistical analysis of the results. According to statistical analysis of the FCV cell culture results of the disinfection tests with Venno Vet 1 Super, Venno FF Super, and Oxystrong FG, the use of suspensions with 40% FBS in a standardized laboratory procedure to simulate dirty field conditions seemed suitable and convenient, since no significant differences (95% level of confidence) were disclosed between tests with FBS and tests with feces. However, these results should be interpreted with caution, as there were cases in which FBS and feces produced statistically significant differences in the treatment effects (during suspension tests with bleach). The statistical analysis of the results of the disinfection tests on NV showed no significant differences in titer reductions between treatments with 1% Oxystrong FG, 5% Venno Vet 1 Super, and 2% Venno FF Super, although 1% Oxystrong FG and 5% Venno Vet 1 Super appeared to be equally more effective than 2% Venno FF Super. Disinfection with 2% Oxystrong FG was significantly more effective for NV, with a probability level of 0.05, than that with either 2% Venno FF Super or 1.2% sodium hypochlorite (7,000 ppm of free chlorine). High contamination densities significantly reduced (with a 99.9% level of confidence) the efficacy of high chlorine concentrations on NV.

DISCUSSION

NV, a group of viruses within the family *Caliciviridae*, are an important cause of gastroenteritis in humans worldwide (3, 25, 27). Although the illness is usually mild and self-limiting, the virus is highly infectious and is a major cause of morbidity. Prolonged outbreaks have been reported, presumably due to long-surviving viral reservoirs and the high environmental stability of the virus, which can remain viable on inanimate surfaces for several days when dried from a fecal suspension (11, 17). Therefore, knowledge of NV inactivation is crucial to ensure public safety, and measures for the prevention and control of NV spread should include the proper disinfection of environmental surfaces.

Since the only cell culture system described for the propagation of NV is difficult to use and unsuitable for large numbers of titration assays (39), a closely related virus, FCV of the genus *Vesivirus*, which can be cultured and plaque assayed (6, 30, 31, 36, 38, 40), was used as a surrogate for NV as previously suggested (4, 6, 13, 31, 36, 40). Using molecular protocols and similar test conditions, a comparative evaluation of disinfectant efficacies on NV and FCV was carried out.

Using defined concentrations and contact times, three of the four disinfectants used in this study were effective on FCV by cell culture and RT-PCR, with inactivation levels of $\geq$99.9%. Venno FF Super, the glutaraldehyde-based compound, failed to adequately affect FCV according to the RT-PCR results, although the infectivity in cell culture was completely abolished.

The RT-PCR-based detection method documented the usefulness of this procedure in terms of significantly reduced detection time and greater sensitivity than that of the cell culture method when applied to inactivated viruses. This increased sensitivity, however, was due mostly to the ability of the assay to detect intact nucleic acid sequences from viruses regardless of their infectivity, not to a presumed greater sensitivity of the PCR over culture. In fact, considering the results of the titration of untreated FCV, the cell culture appeared to be more sensitive than RT-PCR (both conventional and real-time assays), and a titer of 9.35 $\log_{10}$ $TCID_{50}$/ml produced PCR products up to a dilution of 10^{-5} (5 $\log_{10}$ RT-PCRU/ml), corresponding to 4.35 $\log_{10}$ $TCID_{50}$/ml. However, even though molecular protocols commonly yielded positive results with inactivated virus, they successfully mimicked the experimental conditions and interpretation of algorithms of standard culture-based methods. Efforts to modify molecular detection methods so that they only detected infectious virus, adapting the pretreatment with PK and RNase developed by Nuanualsuwan and Cliver (29), were unsuccessful, likely due to the mechanism of action of PK and to the kind of peptide bond hydrolyzed by this enzyme, together with the modes of action of the disinfectants employed. The high titer of FCV used during disinfection tests (8.5 to 9.35 $\log_{10}$ $TCID_{50}$/ml) may also have played a role.

Generally, disinfectant efficacy was strongly influenced by organic impurities (particularly feces), but it was generally possible to compensate for the effect of decreasing efficiency by increasing the concentration of the active substances. It is therefore necessary to test a disinfectant's efficacy, particularly on surrogate viruses, by using appropriate contaminating substances and realistic circumstances so as not to endanger a successful disinfection. In the case of enteric viruses, which are often found in feces in the environment, the use of FBS as a protein load in disinfection testing may underestimate the effects of inhibitory substances in the field.

Using different but comparable RT-PCR protocols as evaluation tools, a good correlation was found between the declines of FCV and NV RNAs after disinfection. However, differences emerged concerning the capsid stabilities of FCV and NV at low pH and in response to protein alkylation, as documented after disinfection by the organic acid and the aldehyde, respectively. It is important that enteric viruses, such as NV, normally survive the harsh environmental conditions of the gastrointestinal tract, such as low pH, the presence of proteolytic enzymes, and high bile concentrations, and can effectively be more resistant than respiratory viruses to environmental factors and inactivation methods (7). Previous studies documented that NV are not completely inactivated even after exposure for 3 h to a pH of 2.7 and that the NV RNA is still fully protected after exposure to pH 2 for 30 min (7, 19, 20, 41).

In contrast, less divergence emerged after virus oxidation or chlorination. Although the comparison of the FCV and NV linear regression analyses from the real-time PCR assays showed similar trends of amplification with nearly parallel patterns, the fluorescence generated with NV amplification (particularly for high viral dilutions) reached a specific threshold detection level slightly earlier than did that generated with FCV. Thus, either the NV nested RT-PCR was slightly more

sensitive than FCV RT-PCR for low virus concentrations or the NV starting concentration was slightly higher than that of FCV. It is not possible to rule out the possibility that these differences were involved (even partially) in the higher NV resistance than FCV resistance registered after disinfection tests. However, although both FCV and NV belong to the same virus family, our results indicate that they may behave differently during disinfection and that NV may be more resistant than FCV to chemical disinfection. The suitability of FCV as a model for NV should be considered with caution, as the biochemical properties of respiratory and enteric caliciviruses may be different. In addition, disinfection studies with viruses "artificially" seeded in body secretions or excretions may overestimate virus inactivation compared with that achieved in the field using the same doses of disinfectants, since indigenous viruses, naturally aggregated and attached to cell debris, are more protected from disinfection, as previously suggested by Tree et al. (41).

In conclusion, according to the PCR results, high concentrations of Venno Vet 1 Super (5%, instead of 1 to 2%, as suggested by the German Veterinary Association [2]) and sodium hypochlorite (1%, with 6,000 ppm of free chlorine, as recommended by the World Health Organization for the disinfection of critical surfaces contaminated with viruses [38]) and not less than 1% Oxystrong FG and 2% Venno FF Super are required for safe disinfection when a calicivirus-related outbreak is suspected. However, results obtained by molecular methods alone need to be interpreted with caution, since while destruction of the viral genome ensures the lack of a residual persisting infectivity, this is also accomplished before genome degradation is complete, and as demonstrated for all treatments on FCV by cell culture, detection of viral RNA underestimates the reduction of viral infectivity.

Based on the results of this study, disinfection with 2% Oxystrong FG for 1 h is recommended for NV decontamination. In contrast to the other disinfectants, the peroxide showed a good correlation between cell culture and RT-PCR results as well as between FCV and NV results. At the same time, it also showed a limited reduction in performance on contaminated surfaces and under "dirty" conditions, making it a good disinfectant for viruses shed in feces. According to the statistical data, disinfection with 2% Oxystrong FG was also significantly more effective on NV than was either 2% Venno FF Super or 1.2% sodium hypochlorite (7,000 ppm of free chlorine).

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